THE EFFECT OF CUPRIC, MANGANOUS, AND FERRIC CHLORIDES UPON CARDIAC EXPLANTS IN TISSUE CULTURE

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Introduction

Considerable material has appeared in the literature concerning the effect of many metallic ions and salts upon various biological functions. It has appeared from the studies of Titus and Cave (1928), Titus, Cave and Hughes (1928) and Elvehjem (1932) that manganese and iron are particularly prominent in numerous rôles, especially in cellular respiration, hemoglobin formation, and nutrition. To introduce a third element, copper likewise is essential to hemoglobin formation regardless of the amount of iron administered to the organism (Titus and Cave, 1928). Further, it was shown by Titus and Hughes (1929) that copper and manganese may be stored in the animal body in such a way as to be effective in the utilization of iron in respiratory pigment formation. Later studies by Locke and Main (1931) have provided additional evidence that iron is essential for the regulation of cellular respiration and that it, together with copper, forms the oxygen-binding nucleus of the respiratory pigments. The absorption of oxygen by the cells is due to a reaction between molecular oxygen and a complex intracellular iron compound which is believed to be a hematin derivative and is termed "the respiratory enzyme" (Warburg, 1923*a*, 1923*b*, 1925, 1926, 1928; Warburg, Posener and Negelein, 1924).

The fact that these metals play a further part in the proper function of the blood is brought out by Meyer and Eggert (1932), who reported beneficial results following the administration of copper and iron combinations in the treatment of secondary anemia, while the liver and liver extracts used in primary anemia have appeared to be effective for reasons other than that they contain high percentages of these metals. On the other hand, the beneficial effects of hepatic therapy in secondary anemia may be due, at least in part, to the presence of these two metals.

The absence of iron and copper from synthetic media results in the slowing up of yeast cell proliferation and the production of atypical cells with low pigment content. This reduction of pigment has been held to be comparable, in certain respects, to the condition of anemia in animals (Elvehjem, 1931).

Leaving the question of iron and copper for the moment and turning to that of manganese, it has been shown that horses and goats, immunized against diphtheria, and exhibiting a constant fall of antitoxin titre, would increase the titre following injections of manganese chloride. Small injections of this salt increased the power of the organism to destroy the bacterial toxins to such a degree that animals so treated were not poisoned by an otherwise fatal dose of toxin. This was particularly true in cases of tuberculosis in mice and guinea pigs (Walbum, 1921, 1924). Although manganese will raise the titre of diphtheria antitoxin, it will not change that of tetanus, nor has it any effect upon agglutinins or hemolysins (Pico, 1924).

The experiments reported in the present article were devised to test the effect of different concentrations of the three metals mentioned upon the growth and longevity of cells in tissue cultures. The 3,000 cultures examined in this study have yielded some rather definite information on the questions of the toxicity of, and the tolerance to, these elements when used singly and in combinations. It became of interest to determine whether a tolerance could be built up for such solutions as proved toxic; and whether a combination of non-toxic, or even beneficial solutions was more beneficial than the constituents used separately.

Technique

All of the cultures used in these experiments were made by the cover-slip-hanging-drop method. The procedures were carried out with aseptic precautions in each instance. All water used was triply distilled in an all-Pyrex-glass apparatus. The plasma was obtained by centrifuging blood drawn from the wing veins of young hens. Embryo juice (33 per cent) was prepared by extracting seven-day, or eight-day chick embryos in Tyrode solution (pH 7.4 to 7.6) containing 0.25 per cent dextrose. In order to increase the potency of the extract, it was allowed to stand twenty-four hours at 40° F. before centrifuging (Carrel, 1913). In the experimental series the metallic salts (Merck's), in sufficient quantities for the proper final dilutions, were added to the embryo juice before mixing. Tissue from the hearts of seven-day, or eight-day chick embryos was planted in a mixture of equal quantities of plasma and embryo juice containing the metallic salt. An equal number of controls, using the same plasma, stock embryo juice, and embryo heart tissue, was run with each series of the metallic chloride cultures. These cultures were incubated at 39° C. and careful daily records were kept on each as long as it remained alive. An average percentage death curve was constructed from the data obtained in the control cultures of all experiments

in this paper and serves as the standard for comparison with the curves obtained from the metallic ion series.

Experimental

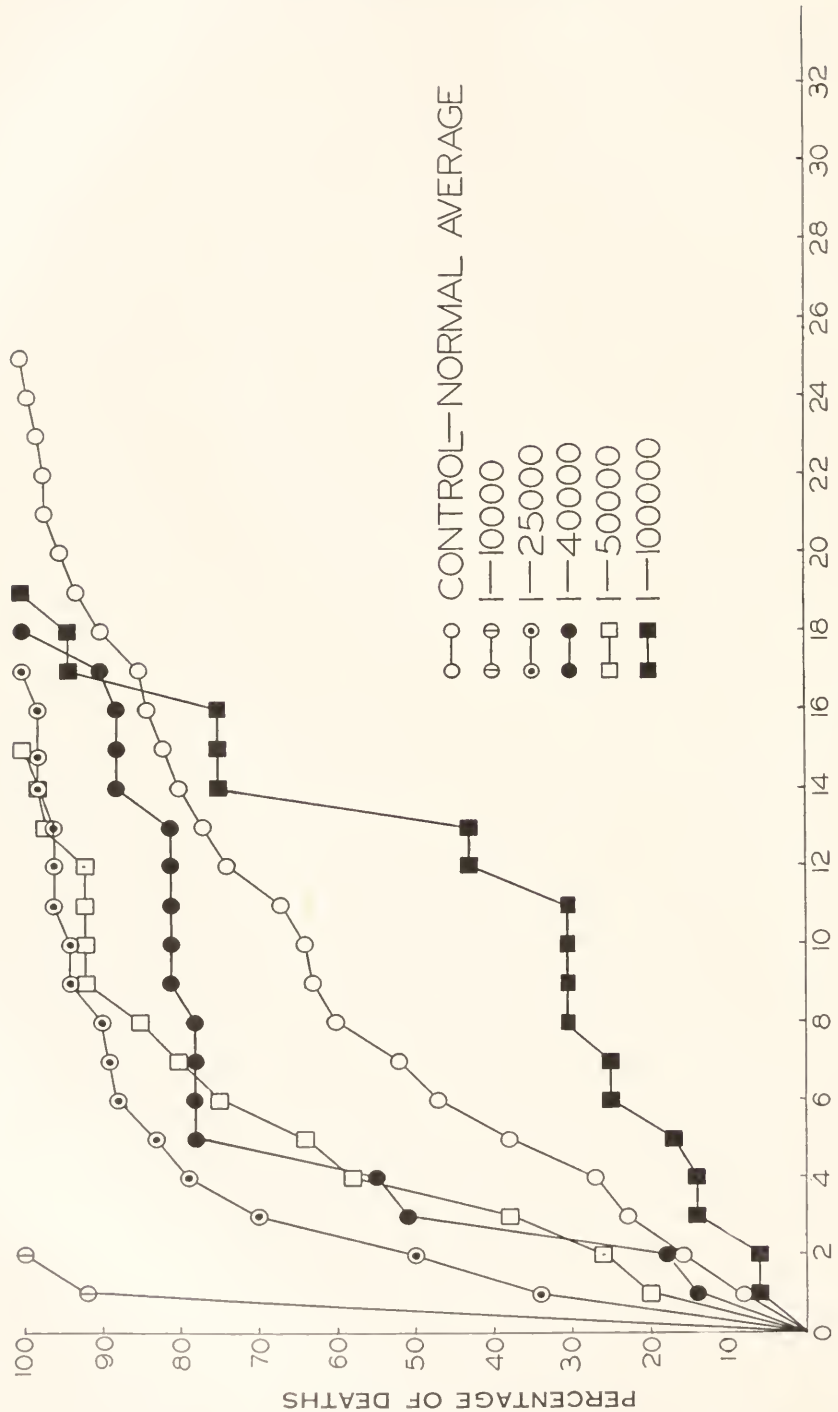
Cupric Chloride Experiments

In these experiments to test the effect of the copper salt, $CuCl_2 .2H_2O$ was added to embryo juice samples in sufficient quantities to make a range of dilutions and normalities, after an equal quantity of plasma had been added, as follows: 1–10,000 (.00117 N), 1–25,000 (.00056 N), 1–40,000 (.00029 N), 1–50,000 (.00023 N), 1–100,000 (.000117 N).

The average death curve of the control cultures climbed rapidly at first and then more gradually until the twenty-fifth day when all cultures were dead. Cupric chloride—1–10,000 (Graph I)—was found to be very toxic to the explants, as evidenced by an almost straight curve; 92 per cent of all the cultures died within twenty-four hours and the remaining 8 per cent within forty-eight hours. In cultures with this concentration of copper, growth was practically inhibited; only occasionally was there any evidence of the migration of fibroblasts. In the 1–25,000 dilution series a fair radial outgrowth of mesenchymal cells was present twenty-four hours after planting. However, the curve rises very rapidly until the sixth day, after which it resembles the control curve. This may be due to either of two factors or a combination of them. It is possible that the tissues became accustomed to the copper salt or, after active proliferation of fibroblasts began, the toxicity of the chloride may have become proportionately less as the cell mass increased.

The curves of cultures planted in 1–40,000 and 1–50,000 dilutions of the copper salt rise rapidly and more or less in company until the fifth day. Then they diverge and the lesser dilution curve flattens out, rising slowly until the seventeenth day. On the other hand, the 1–50,000 dilution curve continues to rise until the ninth day, after which the rise is less acute until the fifteenth day when all cultures were dead. The radial outgrowth of cells in both these dilutions was good, though markedly better in the greater dilution. This fact may account for the different nature of the curve. The possibility, which suggests itself here, is that the life span was shorter because of lack of nutriment.

In the 1–100,000 cupric chloride series, the radial outgrowth of the cells was very marked and exceeded the growth of the controls. This is evidenced by the curve which shows that on the eleventh day only 30.5 per cent of the cultures were dead as compared with 67 per cent of the controls. However, from this point on, the curve rises very



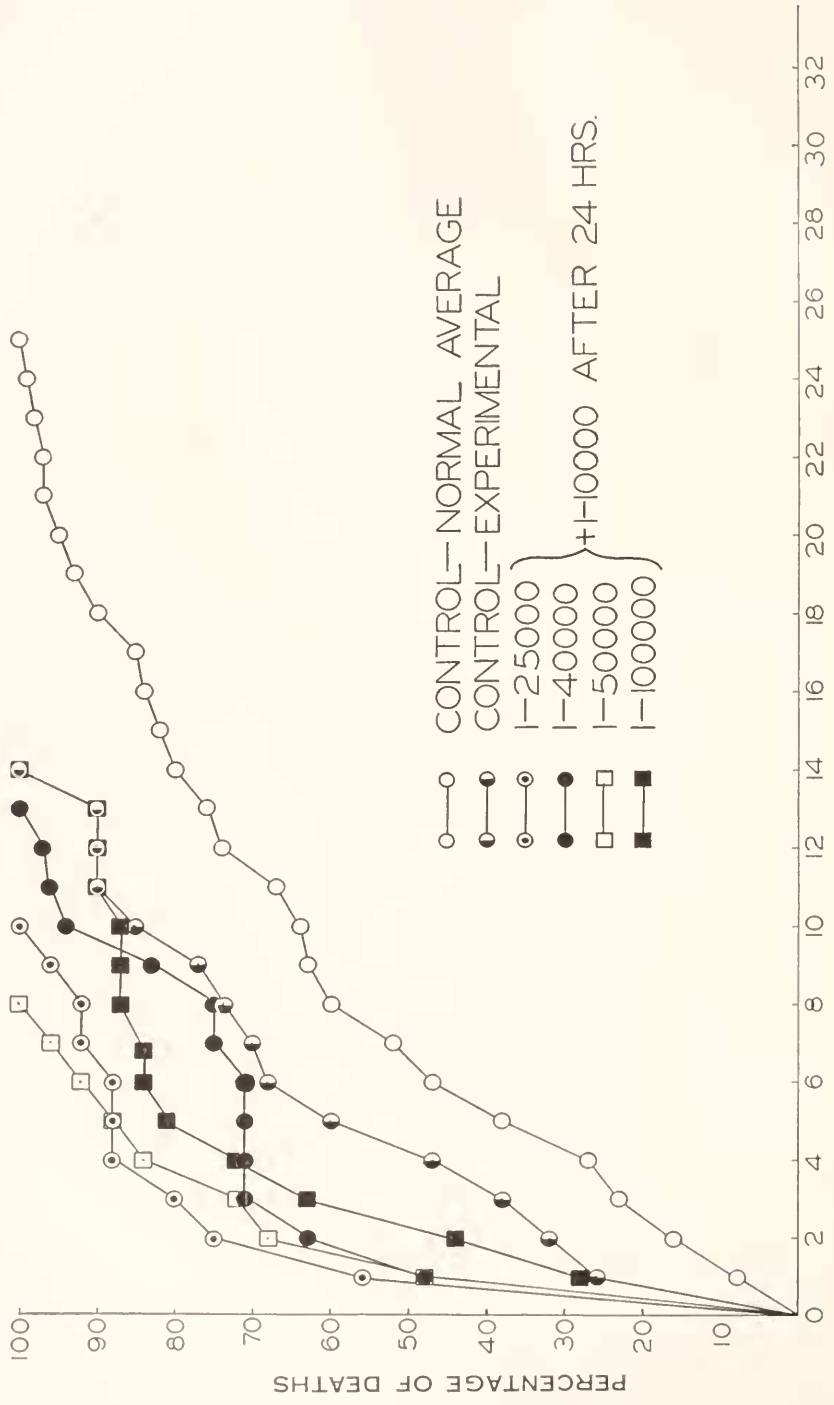
Graph I. Cupric chloride dilutions.

rapidly, possibly because of the toxic effect of the salt together with the lack of nutriment and accumulation of waste products.

A second series of experiments to test a possible tolerance of the cells to cupric chloride was carried out by the administration of a toxic concentration of the metallic salt after a period of adjustment in greater dilutions. Cultures were planted in each of the four salt dilutions: 1–25,000, 1–40,000, 1–50,000 and 1–100,000 and allowed to incubate for twenty-four hours. At the end of this period of preliminary adjustment and growth, each living culture was unsealed, the fluid drawn off and replaced by one drop of 1–10,000 copper chloride embryo juice. This concentration was selected because it had been found to be the most toxic of the solutions employed. Following this treatment the cultures were sealed, incubated as usual, and observed at twenty-four hour intervals until all were dead. The experimental controls for this series consisted of cultures planted in the regular manner without any copper salt added until after the first twenty-four hours of incubation when one drop of 1–10,000 copper chloride embryo juice was added. Thereafter the results were recorded as for the other series and all appear in Graph II.

For the first three days the percentage death curves have much the same shape, rising rapidly. During this interval the toxicity was most marked in the 1–25,000 dilution (80 per cent), about the same for 1–40,000 and 1–50,000 (71 and 72 per cent), less in the 1–100,000 (63 per cent) and least in the experimental control (38 per cent). If one compares the curve of cultures planted in 1–10,000 $CuCl_2$ (Graph I) with the curves of the culture series in the varying dilutions to which 1–10,000 $CuCl_2$ was added after twenty-four hours (Graph II), one notes that the additional copper is tolerated rather well though the life span is much less (a difference of eleven days for 1–100,000 $CuCl_2$ series) for all series compared with that of the normal controls. The experimental controls, which were normal cultures allowed twenty-four hours undisturbed growth and then subjected to copper chloride 1–10,000, died off less rapidly than the cultures planted in the various dilutions and then treated with the toxic dose. The total life span, however, was the same as that of the 1–100,000 dilution cultures in this particular experiment. It seems from this that a very critical period in the life of the explanted tissues occurs within the first twenty-four hours of adjustment and growth.

Another experiment was devised to test whether the age of the culture, before adding the toxic 1–10,000 $CuCl_2$, would influence the tolerance as evidenced by the longevity of the tissue. A large series of cultures was planted in the various dilutions of copper chloride as before



GRAPH II. Cupric chloride tolerance.

and divided into groups. After twenty-four hours incubation a set of vigorous cultures from each dilution series was unsealed and each preparation received one drop of 1–10,000 $CuCl_2$ embryo juice. They were resealed and returned to the incubator. On the second, third, fourth and fifth days a fresh group from each series was similarly treated. By this means the cultures were allowed to establish growths in their respective copper dilutions from one to five days before their susceptibility to the toxic concentration of copper was tested.

The results of this experiment were rather unsatisfactory. However, the series in which the procedure duplicated that of the tolerance experiment just described, i.e., addition of the toxic solution on the day following planting, reproduced the curves of Graph II almost exactly. Two days difference in the life span was the greatest variation and that occurred in the 1–50,000 dilution.

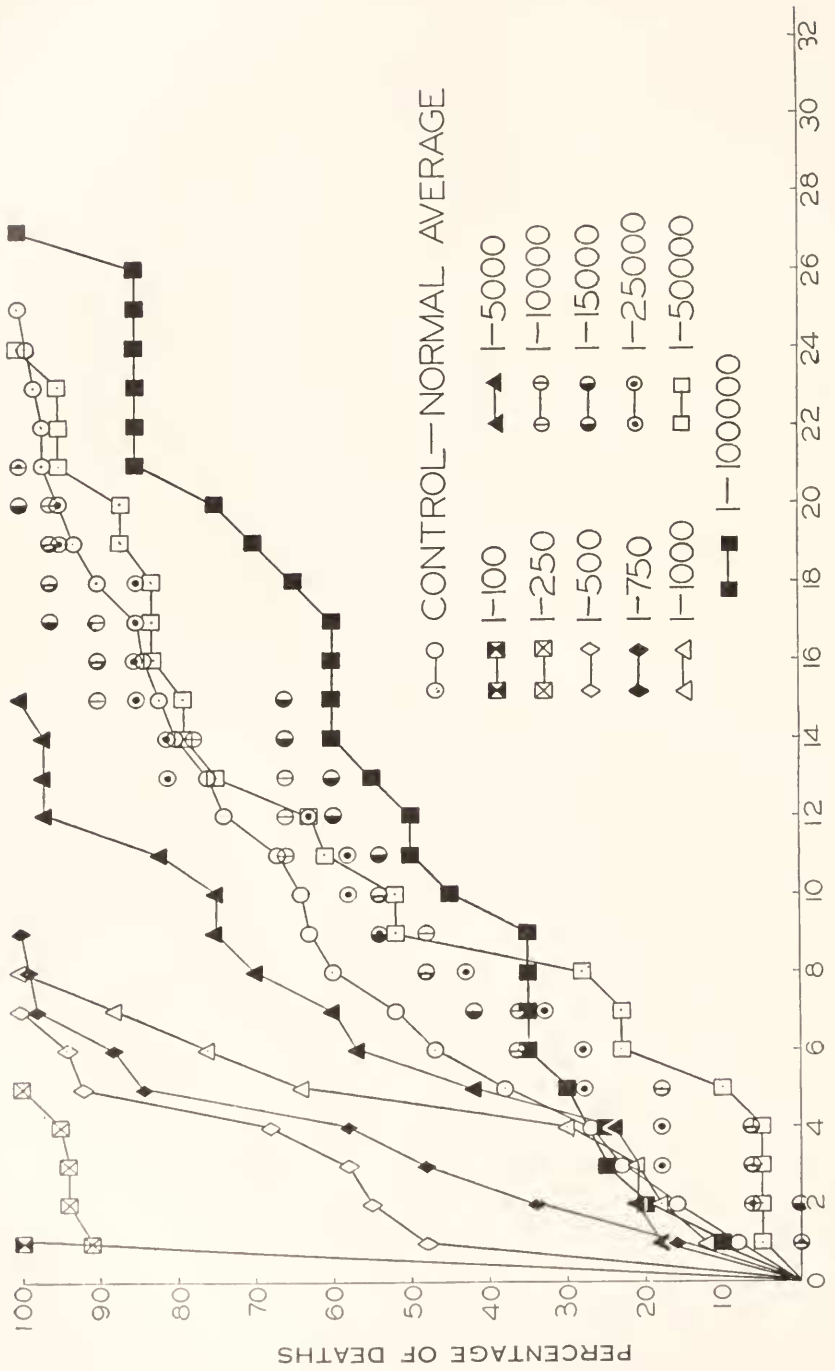
With the exception of the cultures in 1–25,000 dilution, the optimum resistance to an added toxic dose of copper salt, as evidenced by plotting the results, was acquired between the third and fourth days of growth.

Manganous Chloride Experiments

Experiments, conducted in a manner similar to those of the copper series, were carried out with manganous chloride, $MnCl_2.4\ H_2O$, added to the embryo juice. This salt was added in quantities sufficient to form the following final dilutions in which series of cultures were planted: 1–100 (.10 N), 1–250 (.04 N), 1–500 (.02 N), 1–750 (.013 N), 1–1,000 (.010 N), 1–5,000 (.002 N); 1–10,000 (.0001 N), 1–15,000 (.0006 N), 1–25,000 (.0004 N), 1–50,000 (.0002 N), 1–100,000 (.0001 N).

When the percentage death curves were plotted and compared with the average curve of all normal controls, it was found (Graph III) that they fell roughly into three major groupings. The series 1–100 through 1–1,000 showed a marked toxicity of the salt; the 1–5,000 sets were isolated midway between these and the remaining dilutions. The curves of all dilutions from 1–15,000 through 1–50,000 remained below the control curve until the twelfth day after which they fell to one side or the other of this curve. The 1–100,000 dilution curve, from the fourth day onward, was always below that of the control and terminated on the twenty-seventh day, exceeding the control in length by two days.

From these curves it will be seen that the presence of manganous chloride in dilutions of 1–15,000 through 1–50,000 did not markedly influence the behavior of cells in cultures although the life span was decreased one to five days. The cells grew and radial outgrowth was



GRAPH III. Manganous chloride dilutions.

almost as prolific as in the controls The aging process,[1] slower in these cultures than in the controls, was hastened a little after the twelfth day.

Manganous chloride 1–100,000 prolonged the life of cultures for two days as compared with the controls. This in itself was not a significant increase, but in view of the fact that the percentage deaths throughout the series on any one day after the sixth were from 10 to 20 per cent less than for the normal controls, it seemed that manganese in this concentration may have exerted a slight influence in the direction of longevity.

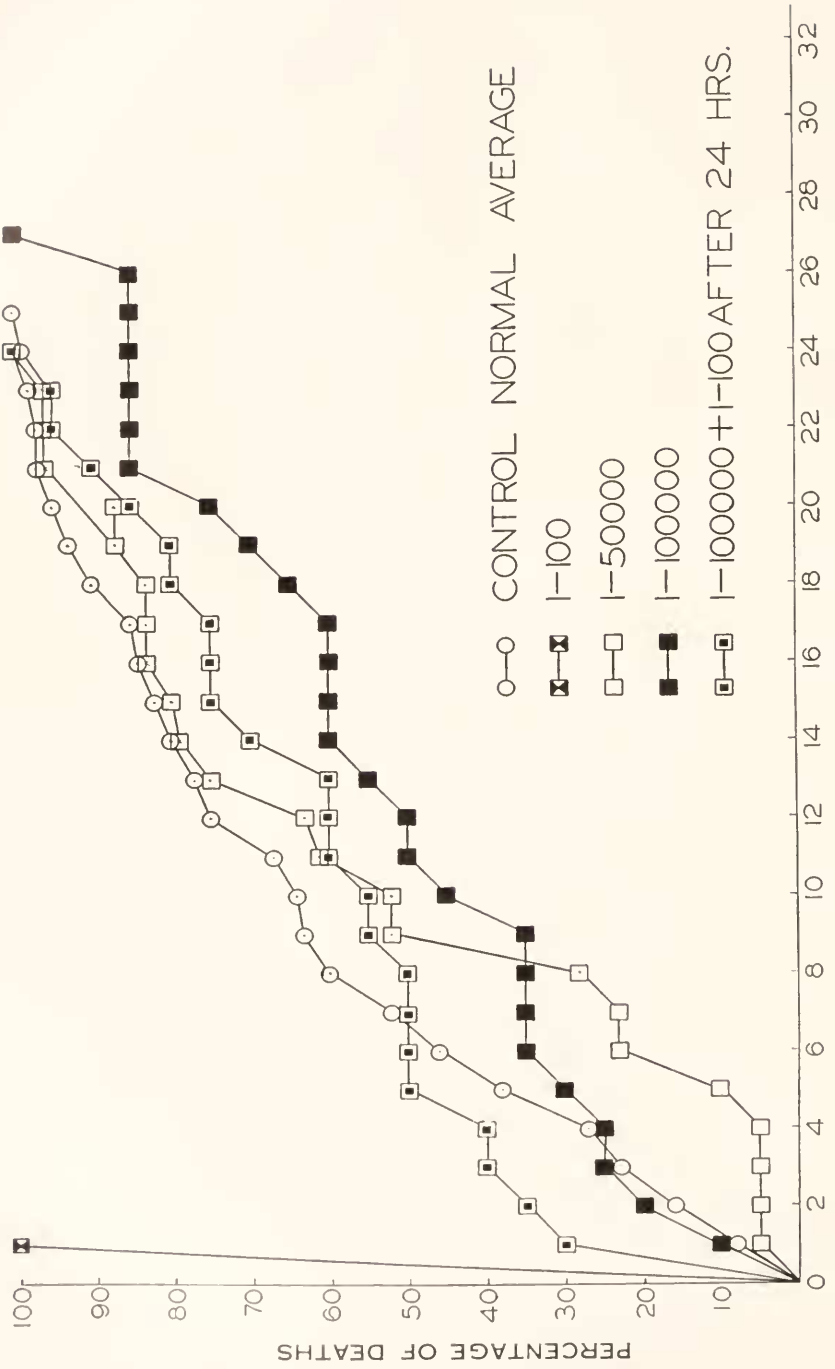
To ascertain whether the cultures would develop a tolerance for manganese, a series of plantings in 1–100,000 dilution were allowed to adjust themselves for twenty-four hours. At the end of that time they were unsealed and to each culture was added one drop of 1–100 $MnCl_2$ embryo juice. Then they were resealed and returned to the incubator. The results of these series showed that the addition of the very toxic solution (see Graph IV) did not cause the death curve to rise precipitously as in the 1–100 dilution series, but rather to rise rapidly until the tenth day and thereafter to follow very closely the curve of the 1–50,000 dilution. It would seem, therefore, that preliminary treatment with a non-toxic manganese concentration protected, to some extent, cultures subjected later to a fatal concentration.

Ferric Chloride Experiments

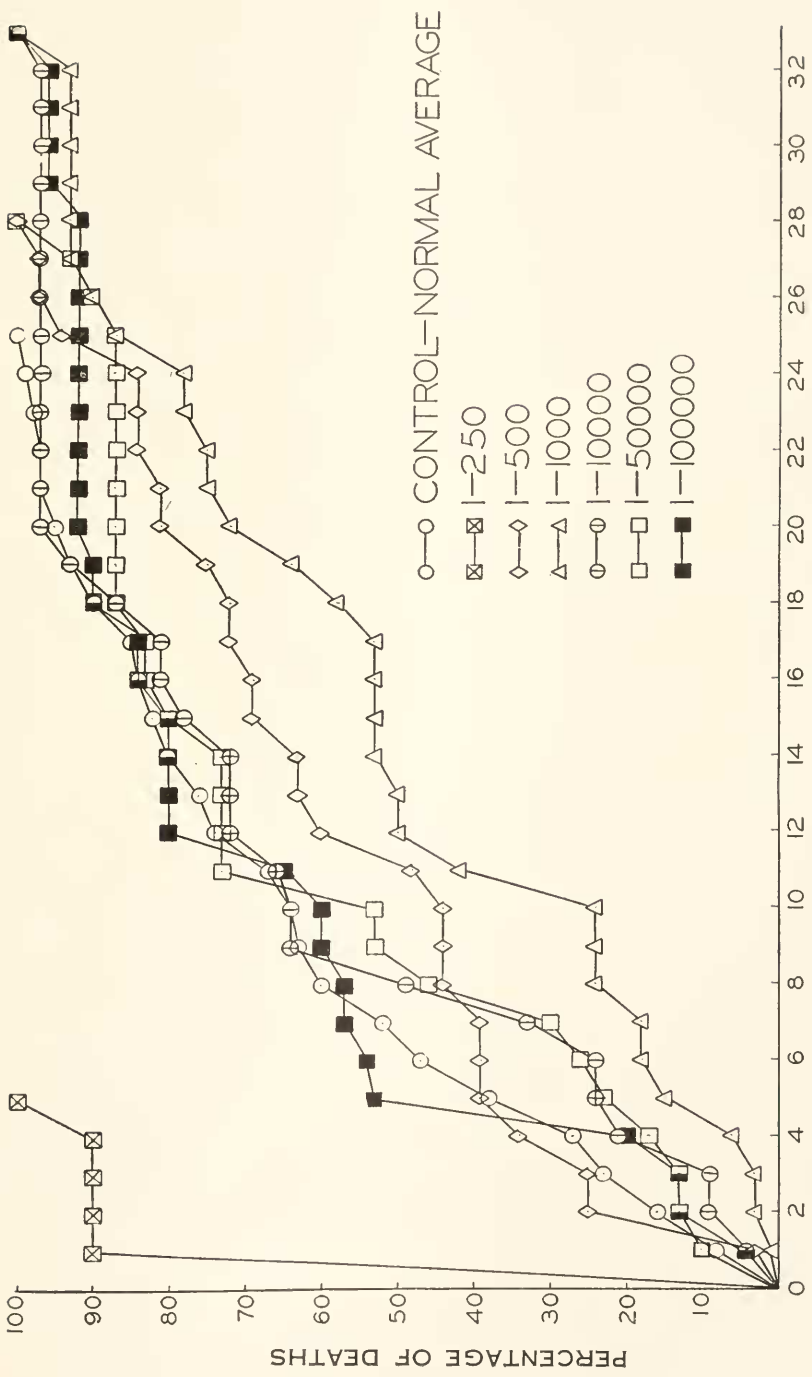
In a manner similar to that used in previous experiments plantings were made in the following $FeCl_3.6\ H_2O$ solutions: 1–250 (.044 N), 1–500 (.022 N), 1–1,000 (.011 N), 1–10,000 (.0011 N), 1–50,000 (.00022 N), 1–100,000 (.00011 N).

The results of these series are shown in Graph V. The dilution 1–250 proved to be highly toxic, 90 per cent of the cultures died in the first twenty-four hours and all of them were dead in five days. The curves of the other dilutions except 1–500 and 1–1,000 followed quite closely that of the average normal control, falling but a short distance above or below it until the eighteenth day when they diverged. With the exception of the 1–250 dilution, all other curves exceeded the normal in duration from three to eight days. Contrary to the results obtained for the copper and manganese chlorides, the 1–1,000 dilution of the $FeCl_3$ proved to be the least toxic of all and the life span of this series was prolonged eight days over that of the controls. This is a significant increase and might suggest that this dilution of ferric chloride was

[1] The addition of potassium permanganate to mesenchymal cells in cultures was found by Lewis (1921) to reproduce, within a few minutes, the degenerative changes that took place gradually in the normal aging and resultant death of cells.



GRAPH IV. Manganous chloride tolerance.



GRAPH V. Ferric chloride dilutions.

beneficial to cultures. However, one observation must be kept in mind in this connection. After tissues were planted in the $FeCl_3$ solutions, a brown precipitate was produced in the medium and in all the iron salt cultures, histiocytes were found in noticeably greater numbers than in either copper or manganese cultures. These phagocytic cells contained quantities of engulfed iron precipitates. Whether the formation of precipitates rendered the iron less toxic or whether the abundance of histiocytes exerted a beneficial influence upon the cultures is not known. As suggestive evidence, only, in support of the latter idea, the senior author noted, in some other experiments using cardiac tissue planted in a medium containing trypan blue, that histiocytic outgrowth was stimulated and that cultures so populated remained in better condition longer than those without an abundance of macrophages.

In order to test the development of tolerance to iron, a series of cultures was planted in 1–1,000 $FeCl_3$ and incubated for twenty-four hours before each culture received one drop of 1–250 iron salt. As in previous experiments of this nature, the preliminary treatment of the cultures with the least toxic dilution of the salt lessened (Graph VI) to a marked degree the toxicity of a fatal dose; although the life span was reduced from thirty-three days (1–1,000 dilution curve) to twenty-six days (tolerance curve) as compared with the five-day span for cultures planted only in 1–250 dilution.

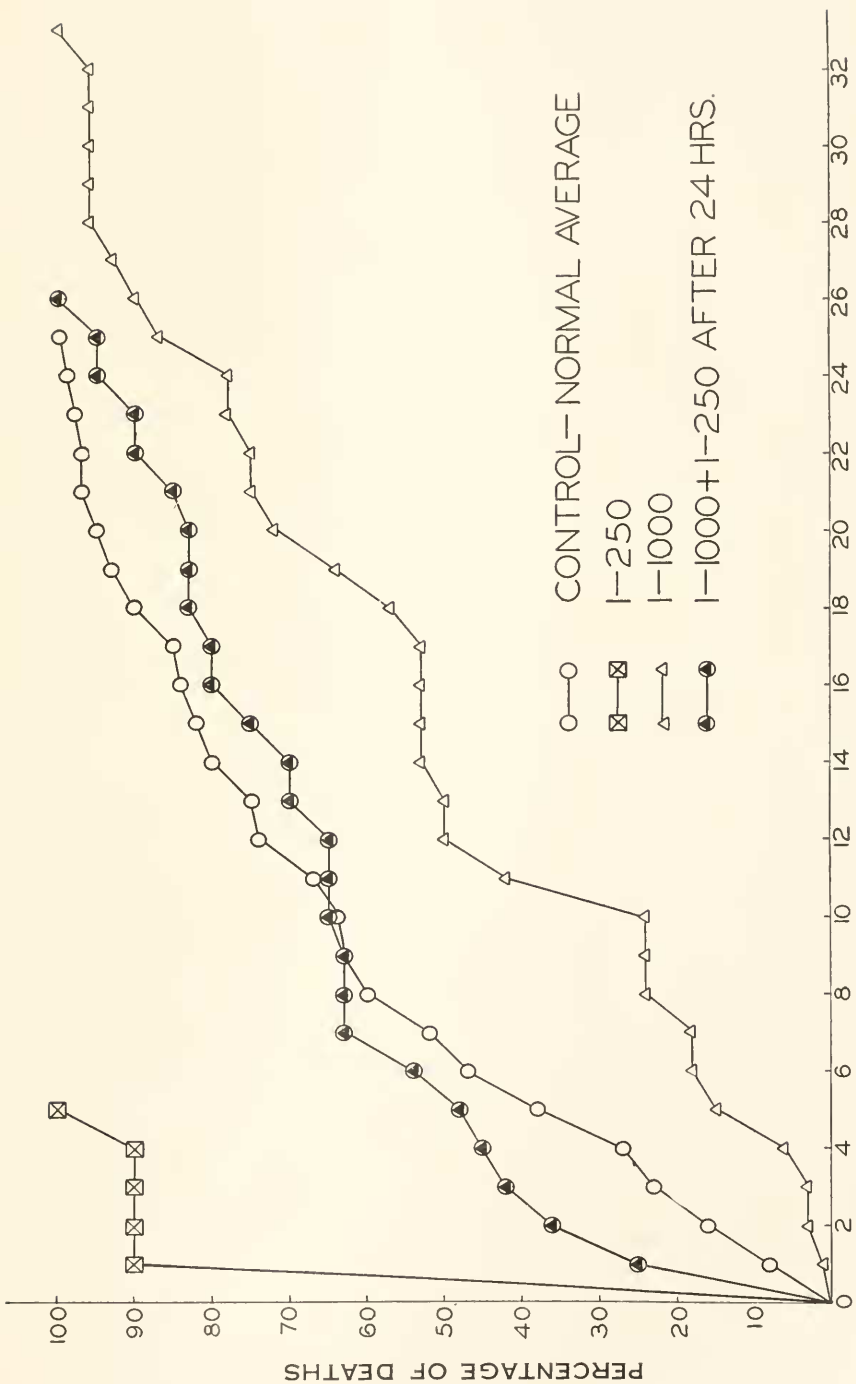
Combination Experiments

In view of the fact that certain dilutions of each of the three metallic chlorides utilized in the preceding experiments were found to be relatively non-toxic, four combinations of the optimum dilutions of the different chlorides were made and cultures were planted in them. The same technique used in the previous experiments was employed here.

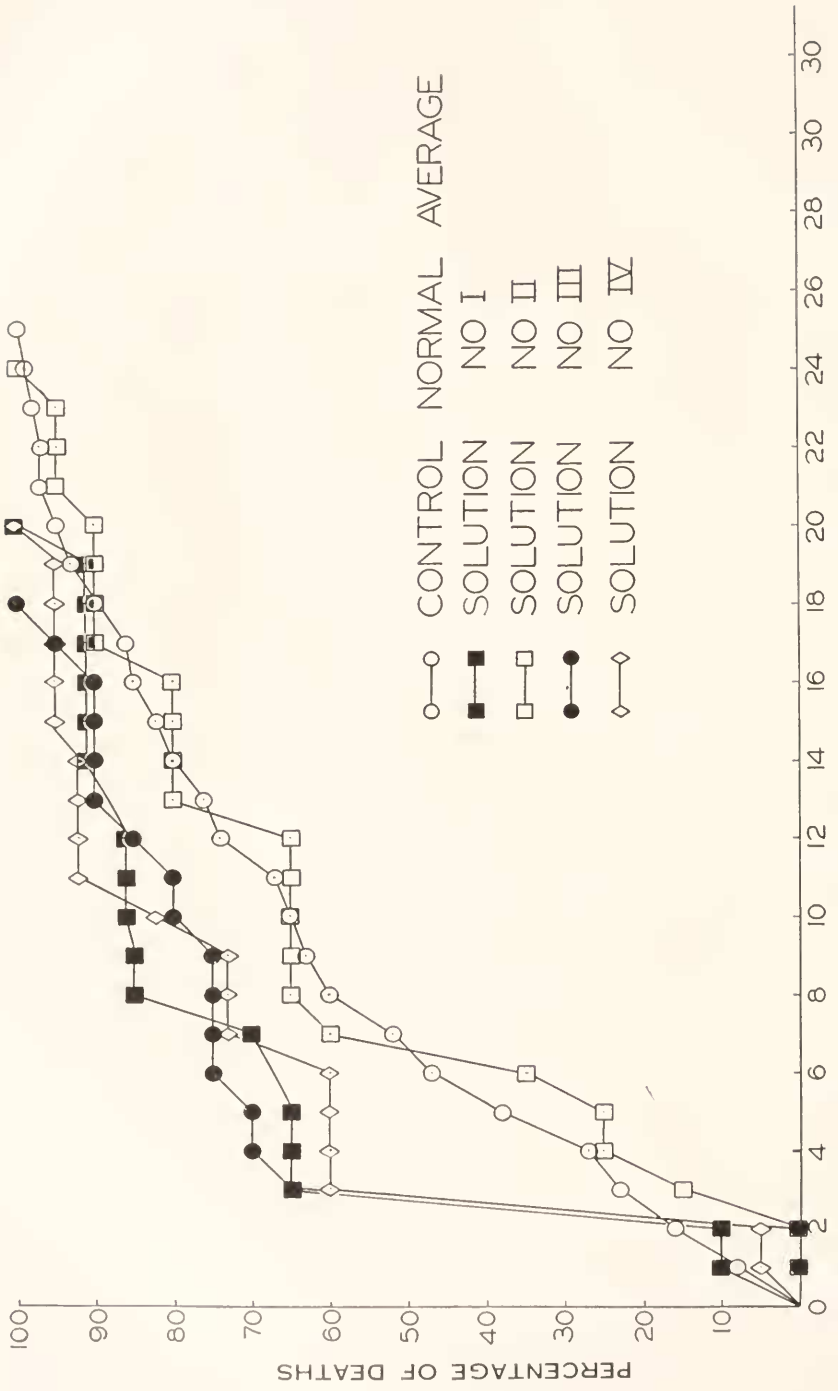
The salt mixtures used were as follows:

Solution 1.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
	(*c*) Manganous Chloride	1–100,000
Solution 2.	(*a*) Ferric Chloride	1–1,000
	(*b*) Manganous Chloride	1–100,000
Solution 3.	(*a*) Ferric Chloride	1–1,000
	(*b*) Cupric Chloride	1–100,000
Solution 4.	(*a*) Manganous Chloride	1–100,000
	(*b*) Cupric Chloride	1–100,000

Solutions 1, 3, and 4 were the most toxic and caused sudden death of 60 to 65 per cent of the cultures by the third day (Graph VII); those remaining died by the eighteenth to twentieth days as compared with a life span of twenty-five days for the normal controls. Solution 2 was



Graph VI. Ferric chloride tolerance.



GRAPH VII. Combination solutions.

the least deleterious; its curve followed closely that of the control and the life span of the series was only one day less. None of the salt mixtures had any advantage over the single optimum dilutions of the respective salts composing it. It was noted again, however, that wherever iron was present in the solutions, the cultures contained a greater number of histiocytes than otherwise.

Summary

Throughout these experiments there were certain characteristic reactions in the mesenchymal cells growing in the various dilutions of the metallic chlorides used. The nuclear membrane and nucleoli were generally a little more sharply defined than in the cells of the controls. Accumulation of vacuoles, first about the nucleus and finally throughout the cytoplasm, took place rapidly in the toxic salt solutions. At times the cells were so thoroughly occupied by fatty looking vacuoles that they bulged, rounded up and floated away. Similar accumulations of vacuoles occurred in the controls but their appearance was gradual and they only assumed great numbers as the cultures aged.

Optimum dilutions of the metallic salts, in which a depression of the daily death rate and an increase in the life span of the cultures were noted, delayed the degenerative changes in the cells beyond the time when they usually made their appearance in the controls.

Of these solutions, cellular outgrowth was actually stimulated in that of copper chloride, the death rate was depressed in that of manganese chloride, as also in that of iron chloride. Furthermore, the life span was increased in the latter a significant number of days. This effect possibly may have been brought about indirectly as a result of histiocytic stimulation.

Tissues could be protected to some extent from the action of a fatal dose of these three metallic salts by first growing the cells in the respective optimum dilutions.

No beneficial effects were obtained by growing tissues in various combinations of the optimum dilutions of these salts.

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